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The goal of this proposal is to identify and characterize proteins involved in signaling by the estrogen receptor (ER), a transcription factor involved in many human breast tumors, using genetic strategies. Through dosage suppression analysis, we have isolated a gene that when overexpressed dramatically increases ER transcriptional activity. The gene is the yeast homologue of the human p23 protein, a component of the molecular chaperonin complex bound to many unliganded steroid receptors. The cellular distribution of p23 is mostly cytoplasmic, however, this pattern is altered upon expression of ER, whereupon p23 colocalizes with ER in the nucleus. This colocalization was seen in the absence of estrogen: addition of estrogen results in redistribution of p23 to the cytoplasm. Our work suggests that p23 is a positive regulator of ER, and therefore a possible means by which to control ER function and stop breast cancer growth.

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5) Introduction

Estrogen is a steroid hormone responsible for the proper function of multiple mammalian physiological processes. In addition to its central role in reproduction, estrogen also affects the cardiovascular, skeletal, immune, and nervous systems(1,2,5). Estrogen has also been implicated in the initiation and maintenance of breast and ovarian cancers(9,10).

The estrogen signal is mediated by the estrogen receptor (ER). In the absence of estrogen, the ER is found predominantly in the nucleus, existing as a monomer bound to a number of proteins, including heat shock protein hsp 90, p23 and the immunophilins(6,7, 8). The resulting steroid-free "aporeceptor complex " inhibits ER's transcriptional activation, while keeping ER in a conformation amenable for steroid binding. Upon binding estrogen, the ER dissociates from the aporeceptor complex and dimerizes and recognizes specific DNA sequences, called estrogen response elements (ERE), within the promoter regions of estrogen responsive genes. Once bound to an ERE, the ER modulates transcription of the linked gene through interaction with the transcriptional machinery, although the exact mechanisms by which this occurs has not yet been determined(1,4).

Although many aspects of ER signaling remain to be understood, it appears that those proteins essential to ER function are conserved to such an extent among eukaryotes ER signaling can be faithfully recapitulated in yeast (*Saccharomyces cerevisiae*)(3). Yeast do not contain endogenous ER, however, the mechanism of eukaryotic gene transcription appear to be sufficiently conserved so that ER able to function in yeast in a manner analogous to that in mammalian cells. It has been established that human ER expressed in yeast can bind DNA in response to estrogen, subsequently activating transcription from EREs located in promoters upstream of reporter genes.

The genetic capabilities of yeast make it a powerful system in which to study ER function. Our overall goal is to use genetic approaches to identify proteins that affect ER function within this system to define the mechanism of signal transduction and transcriptional regulation by the receptor. We expect that the characterization of these proteins will lead to a greater understanding of the ER signal transduction pathway, and ultimately, to the identification of mammalian counterparts involved in ER function. Although the study of ER function in yeast is unlikely to lead to the understanding of the varied estrogen-induced effects seen in mammalian cells, it is a useful model for trying to determine the basic mechanism of ER signal transduction.

Factors that interact functionally with ER can be identified in yeast through dosage suppression analysis. In this approach, mutant ER proteins that display altered ability to activate transcription can be used as substrates in a dosage suppression screen to isolate

yeast genes that are capable of overcoming this discrepancy in activity. Overexpression of factors important for ER function can in principle overcome the mutational block by favoring the interactions that facilitate ER function. The advantage of this procedure is that it results in the direct cloning of genes of interest.

Using as our substrate, a mutant ER with a reduced ability to bind ligand (ER G400V), we have isolated a large genomic fragment capable of greatly increasing ER's ability to activate transcription in response to hormone. This high copy suppressor of ER is the yeast homologue of the mammalian p23 protein (yph23), a molecule previously described as a member of the aporeceptor complex of several steroid receptors. In this progress report, we provide evidence of both functional and physical interaction between the yeast p23 protein and ER. Our findings indicate that p23 is a positive regulator of the ER pathway, acting as a member of the aporeceptor complex of ER in yeast.

6) Body

Yeast Dosage Suppression Screens

One method to identify factors that affect ER function in yeast is suppressor analysis. In this approach, a mutant ER protein, displaying a reduced ability to activate transcription, is used as a substrate to isolate yeast gene product(s) that are capable of overcoming this reduced receptor activity. The simplest such strategy is dosage suppression: suppression of a mutant phenotype accomplished by overexpression of a wild type yeast gene. Overexpression of factors important for ER function can in principle overcome the receptor mutation by favoring the interaction between ER and such factors, thus reconstituting ER transcriptional activity. A major advantage of dosage suppression analysis is that it results in the direct cloning of the gene(s) that affect ER activity. Another inherent advantage of this approach is that the small size of the yeast genome requires the screening of only several thousand colonies to assay every potential yeast gene versus the millions that would be necessary to screen the larger mammalian genome. The recently completed sequencing of the yeast genome also makes possible the rapid identification of genes residing in the suppressor clone by simply matching the flanking sequences to the yeast genome database. Characterization of the isolated suppressor protein's normal function in yeast as well as in ER signaling is greatly facilitated by the relatively simple construction of yeast strains that lack the gene of interest. Thus, yeast dosage suppression analysis provides a simple and sensitive approach to the identification and isolation of factors that functionally interact with the receptor.

The ER used in the dosage suppression screen contains a mutation that replaces a glycine with a valine at residue 400 (G400V ER) within the steroid binding domain. This

receptor mutation results in decreased steroid hormone binding by ER, with a corresponding reduction in ER's ability to activate transcription at particular concentrations of estrogen. We chose this mutation as a substrate for the screen because G400V ER affects an early step in the ER pathway, namely steroid binding, and has the potential to result in the isolation of the greatest number of proteins important to ER function: factors important for either steroid binding or transcriptional activation will be capable of suppressing the G400V ER phenotype.

We tested the function of G400V ER in yeast and found that this receptor mutant exhibits a decreased ability to induce transcription when compared with wt ER, presumably due to its decreased ability to bind ligand. As seen in Figure 1, G400V ER requires a hundred-fold increase in ligand concentration before receptor transcriptional activation is observed, as compared to wt ER. At saturating ligand concentrations, however, G400V is able to reach the same maximal activity as the wt ER, suggesting that once the block to steroid binding is overcome, the receptor is able to act as efficiently as wt ER in entering the various interactions, both protein-DNA and protein-protein, that are necessary for transcriptional activation. Exploiting this phenotype, we carried out a dosage suppression screen using a high copy yeast genomic library to identify yeast proteins important for ligand binding or transcriptional activation.

The screen was carried out under conditions where yeast colonies containing the wt ER are dark blue, while the G400V ER-expressing yeast appear white (Figure 1B). Yeast strains containing G400V ER and an estrogen-responsive β-galactosidase reporter gene were transformed with a high copy yeast genomic library and assayed under these conditions. Dark blue colonies were considered to be potential suppressor candidates.

A total of 5622 transformed colonies, which represents approximately one-half of the yeast genome, were screened in an attempt to find suppressors of the G400V ER mutation. Thirteen potential suppressor colonies were isolated, but in only five was the suppression shown to be linked to the library plasmid. One library plasmid greatly exceeded the level of suppression the others exhibited, and this suppressor became the focus of our analysis. This plasmid, termed 4.3, was found to dramatically suppress the G400V ER phenotype, increasing its activity ten-fold (bringing G400V ER activity to one third the level of the wt ER) (Figure 1C). A search of the yeast genomic database, using the flanking sequences of the insert, revealed the suppressor clone to be an 8147 base pair genomic fragment with several open reading frames (ORFs) (Figure 2). At the 3' end is a truncated ORF, YKL 516, which encodes a putative protein kinase. Another, complete ORF has high homology to a known protein: the YKL522 gene is a mitochondrial ADP/ATP carrier protein homologue. However, the suppressor is most likely to be one of

the three complete open reading frames that code for unknown proteins: YKL 525, YKL520 and YKL518.

We constructed several different deletion constructs of the 4.3 plasmid in order to identify the suppressing ORF. These constructs were subsequently assayed for their ability to increase G400V ER transcription. As seen in figure 2, the suppression of the G400V ER phenotype correlated with the presence of ORF YKL 518, indicating that it was the responsible ORF.

A search of the Swissprot database revealed YKL 518 to be the yeast homologue of human p23 protein (yhp23). Although its specific function of p23 is not known, studies *in vitro* suggest that p23 is crucial to the stability of the aporeceptor complexes. Removal of p23 greatly inhibits the formation of both PR and GR aporeceptor complexes, implicating p23 as an important "organizer" of the this complex. More recent work has shown that p23 is a molecular chaperone, interacting with nonnative proteins, suppressing their aggregation, and maintaining them in an intermediate, folding-competent conformation. Thus, these studies suggest that p23 is a molecular chaperone, and may therefore function as such within the aporeceptor complex.

Affects of yeast p23 overexpression on ER and GR transcriptional activation.

Having identified the suppressing ORF as yeast p23, we constructed yeast strains that overexpress a p23 containing a HA-epitope in the presence of either G400V ER, wt ER or GR, along with a reporter plasmid containing β-galactosidase under control of the appropriate hormone response element, ERE or GRE, respectively, and measured receptor transcriptional activity as a function of yph23 overexpression. As seen in figure 4A, yeast overexpressing the HA-tagged yph23 of increases G400V ER by 4 fold, without affecting ER protein levels (Figure 3A and D). Overexpression of yph23 also increases the activity of both wt ER and GR, resulting in a greater than 50% increase in transcriptional activation for either receptor (Figure 3B and C). The ability of yph23 to functionally interact not only with the G400V ER mutant, but also with wt ER and GR, strongly implicates yph23 as a member of the normal signaling pathway of steroid receptors in yeast.

Overexpression of yeast yph23 increases ligand binding by G400V ER

In light of p23's role in aporeceptor complex formation, and given the nature of the G400V ER mutation, we decided to examine whether suppression of the G400V ER phenotype was a result of increased ligand binding by the ER in the presence of overexpressed yph23. Steroid binding by G400V ER in the presence and absence of yph23 overexpression was measured *in vivo* (Figure 4A). Estrogen binding was assayed

by incubating both yeast strains for one hour in media containing [^{3}H]-17 β-estradiol and subsequently washed three times to remove unbound ligand. The amount of bound estradiol to G400V ER was measured by scintillation counter. As a negative control, yeast expressing GR were assayed in parallel, allowing us to determine background estradiol binding: these GR values were subtracted from the G400V ER values to determine the amount of ligand bound specifically to G400V ER.

At both 10^{-7} and 10^{-6} [^{3}H]17-β-estradiol concentrations, elevated yph23 levels resulted in an increase in estradiol binding by G400V ER (Figure 4A and 5B). At a concentration of 10^{-6} 17-β-estradiol, G400V ER bound over three times more ligand in the presence of overexpressed yph23. This pattern was more pronounced at the lower concentration of 10^{-7}, where yph23 overexpression increased ligand binding over five-fold. Western blots of the corresponding yeast cultures indicate that this result is not a function of increased receptor levels (Figure 4C). These two results would seem to suggest that the importance of yph23 to G400V ER ligand binding is inversely proportional to the concentration of hormone present. As a correlation, we might therefore expect that p23's importance to G400 V ER ligand binding would be greater at lower, more physiological, levels of hormone.

Interestingly, little effect of yph23 overexpression was seen on wt ER's ligand binding at either concentration (data not shown). We believe this is attributable to the difference in inherent binding efficiencies of the two proteins. As was shown in Figure 1, G400V ER requires a hundred-fold increase in ligand concentration to reach the same level of transcriptional activation displayed by wt ER. Correspondingly, an effect of yph23 overexpression on wt ER ligand binding might only be expected at concentrations lower than those assayed here. Consistent with this interpretation are the transcriptional assays of Figure 3B, which show that wt receptor function is enhanced by overexpression of yph23.

ER and yph23 co-localize within the nucleus of yeast.

Given human p23's presence in the aporeceptor complexes of PR and GR, and having shown that yph23 functionally interacts with ER within the yeast system, we proceeded to determine if yph23 and ER physically interact. We reasoned that, if yph23 is a member of the ER aporeceptor complex in yeast, then it could be expected to co-localize with ER in the nucleus. In order to determine the pattern of yph23 distribution *in vivo*, we created a yph23-GFP (green fluorescent protein) fusion protein by cloning the GFP at the extreme carboxy terminal of the yph23 protein. Importantly, this yph23-GFP fusion protein was functional and also able to suppress the G400V ER phenotype (data not

shown). The ability of the fusion protein to increase G400V ER activity establishes that the GFP moiety does not affect p23's ability to functionally interact with G400V ER.

Having established a functional interaction between yph23-GFP and G400V ER, we constructed several yeast strains which coexpressed yph23-GFP with G400V ER, wt ER, and GR. As seen in Figure 5, the distribution of yph23-GFP in the absence of any steroid receptor expression is largely cytoplasmic. Interestingly, upon coexpression of G400V ER, yph23-GFP becomes localized predominantly to the nucleus, thus co-localizing with G400V ER (Figure 5B). The same localization pattern was observed when yph23-GFP was coexpressed with wt ER (Figure 5D). Importantly, this pattern of nuclear localization was not seen when G400V ER was coexpressed with just the GFP protein, indicating that yph23 alone is responsible for the localization of the fusion protein to the nucleus (Figure 5C). Additionally, when cells coexpressing wt ER and yph23-GFP were incubated in 17-β-estradiol prior to fixation, no nuclear localization of yph23-GFP was observed: the pattern of expression in these cells resembled that of yeast expressing no ER at all, suggesting that yph23 is released upon estradiol binding (Figure 5E). As a final control, coexpression of GR, a steroid receptor that exists outside the nucleus in the unbound state, did not result in nuclear localization of yph23-GFP (Figure 5F). Instead, a more extreme cytoplasmic expression pattern was seen, where no signal corresponding to the nucleus was visible. Thus, the above-described patterns of yph23 distribution are entirely consistent with the suspected role of yph23 as a member of the aporeceptor complex of both ER and GR.

7) Conclusion

We have identified the yeast homologue of the human p23 gene as a high copy suppressor of the G400V ER phenotype. The p23 protein is a member of the steroid receptor aporeceptor complex. In yeast, overexpression of yph23 results in a greater ligand binding by G400V ER. This increase in steroid binding, in turn, results in a ten-fold increase in G400V ER-dependent transcription. This effect on activity is not limited to the mutant ER, as overexpression of yph23 was similarly shown to increase the transcriptional activity of both the wild type estrogen and glucocorticoid receptors as well. In addition to these functional assays, subcellular localization studies using a yph23-GFP fusion protein have demonstrated that yph23 relocalizes from the cytoplasm to the nucleus upon coexpression of ER, and that this nuclear localization of yph23 can be reversed by the addition of 17-β-estradiol. This nuclear re localization of yph23 was not seen on coexpression of GR, a nuclear receptor which, in the unliganded state, exists solely in the

cytoplasm. Thus, our findings demonstrate that yph23 can both functionally and physically interact with ER *in vivo* and suggets that p23 is an important component of the ER signaling pathway.

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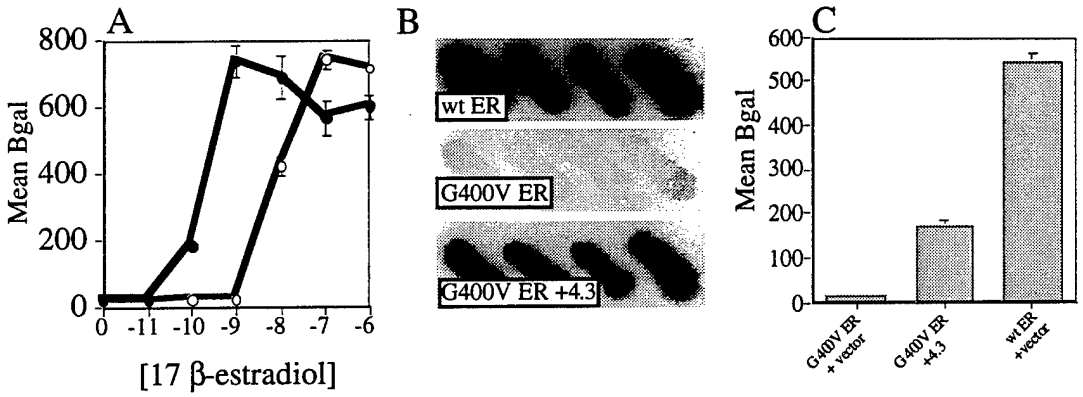

Figure 1. Dosage suppression analysis of ER (G400V).

A). Graph depicts the level of transcriptional activation exhibited by the wt ER (filled circles) and G400V ER (open circles) as a function of β-estradiol. The yeast strain W303a was transformed with 1) a galactose inducible vector expressing the wt ER and mutant G400V ER and 2) a reporter plasmid contain the β-galactosidase gene under the control of an estrogen responsive element (ERE). Cells were grown in selective media containing 2% galactose and 1% raffinose. β-estradiol was added to the media as 10^3-fold stocks in ethanol and β-galactosidase activity was determined 8 hours later. Note that G400V ER requires a hundred-fold increase in ligand concentration before receptor transcriptional activation is observed, as compared to wt ER. Exploiting this phenotype, a dosage suppression screen was performed at a β-estradiol concentration of 10^{-9}M. B). Dosage supression screen. Yeast strains containing G400V ER and an estrogen-responsive β-galactosidase reporter gene were transformed with a high copy yeast genomic library and assayed at 10^{-9} M β-estradiol, conditions where yeast colonies containing the wt ER are dark blue, while the G400V ER-expressing yeast appear white. Dark blue colonies were considered to be potential suppressor candidates. Transcriptional activity of yeast colonies containing the wt ER, ER G400V and ER G400V containing the high copy suppresser 4.3 on galactose-X-GAL indicator plates containing 10^{-9}M β-estradiol. C). Transcriptional activation of wt ER, ER G400V and ER G400V in the presence and absence of the suppresser 4.3. Wt ER and ER G400V mutant in the absence (vector) or presence of the high copy suppresser 4.3 were assayed for β-galactosidase activity in the presence 10^{-9}M β-estradiol.

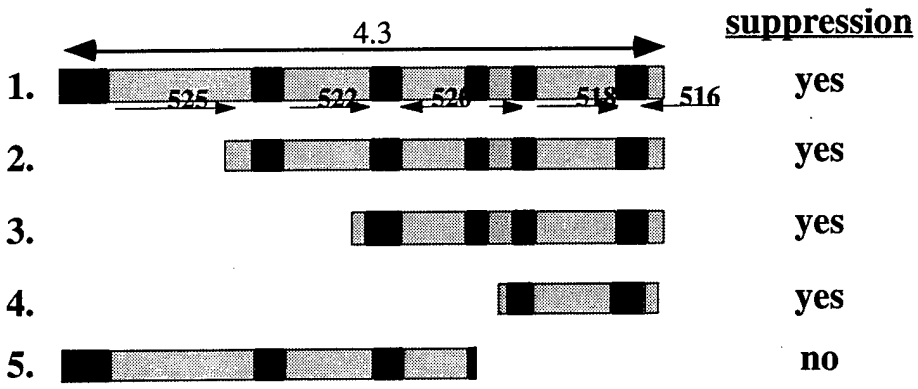

Figure 2. Suppression of ER G400V by YKL 518.
A schematic representation of a fragment of the yeast genome containing the ER G400V suppressor, 4.3. Speckled gray areas show the open reading frames (ORFs) contained within the suppressor, 4.3. Arrows denote the direction of transcription of these ORFs. Four of the genes code for complete proteins, YKL 525, YKL 522, YKL 520, YKL 518. One gene, YKL 516 is truncated at its 5' end and does not code for a protein. Deletion constructs were generated and tested for their ability to suppresses the ER G400V phenotype. ER G400V mutant in the presence of each fragment were assayed for β-galactosidase activity in the yeast strain W303a at 10^{-9}M β-estradiol. Fragments that suppressed are denoted by "Yes", whereas fragments that did not affect suppression are designated "No". Suppression of ER G400V localizes to the YKL 518 gene, which encodes the yeast homologue of the human p23 protein (yhp23).

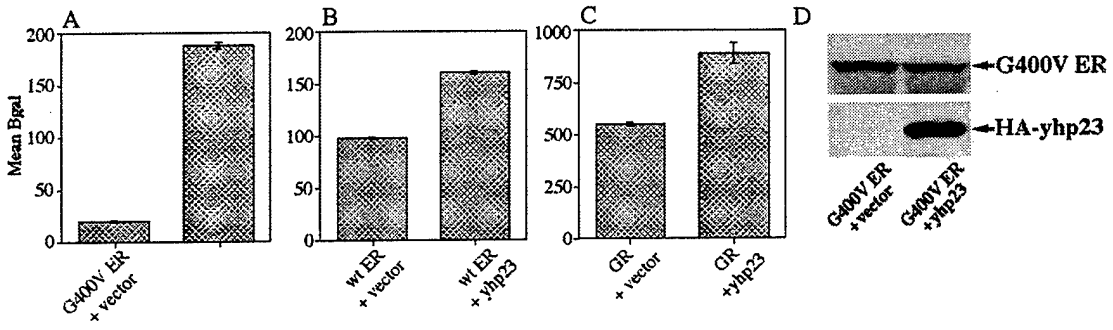

Figure 3. Effect of yhp23 overexpression on steroid receptor activity. Overexpression of yhp23 increases steroid receptor transcriptional activity. (A) ER G400V (B) wt ER, (C) and glucocorticoid receptor (GR), in the absence (vector) or presence of an HA-epitope tagged version of yhp23 in a high copy expression vector were assayed for β-galactosidase activity in the yeast strain W303a in the presence 10^{-8}M β-estradiol, for ER and 10-6M DOC for GR. D) The level of ER protein is not altered by yhp23 overexpression. Whole cell extracts were analyzed from the strain expressing ER G400V in the presence and absence of yhp23 by immunoblotting for ER, using an ER-specific rabbit polyclonal antiserum (top panel), and for HA-tagged yhp23, using anti-HA mouse monoclonal antibodies (bottom panel).

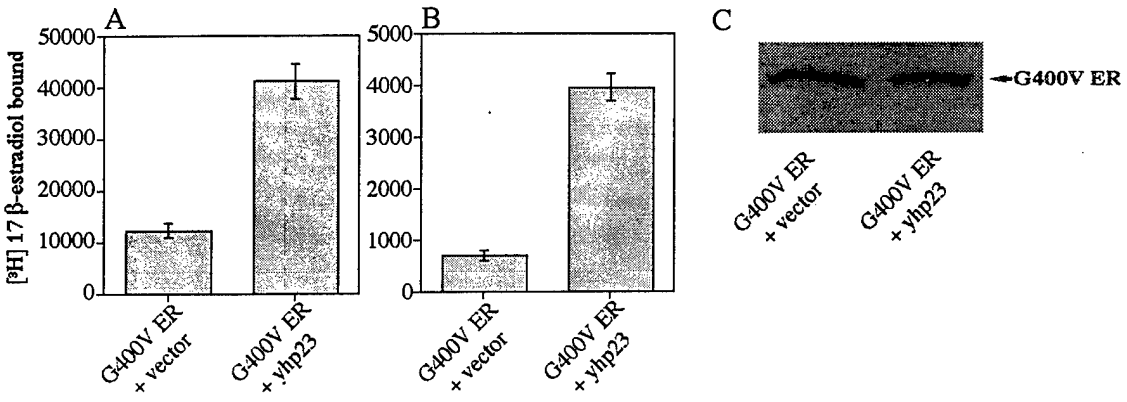

Figure 4. Overexpression of yhp23 increases steroid binding by G400V ER *in vivo*. A) Steroid binding *in vivo* by G400V ER in the presence and absence of yhp23. Estrogen binding was assayed by incubating yeast strains containing G400V ER for one hour in media containing (A) 10^{-7} and (B) 10^{-6} [^{3}H]17-β-estradiol. Cells were subsequently washed three times to remove unbound ligand and the amount of bound [^{3}H]17-β-estradiol was measured by scintillation counter. To determine background estradiol binding, yeast expressing GR were assayed in parallel: these GR values were subtracted from the G400V ER values to determine the amount of [^{3}H]17-β-estradiol bound specifically to G400V ER. C). Protein levels of G400V ER are not affect by yhp23 overexpression. Whole cell extracts were analyzed from strain expressing ER G400V in the presence (vector) and absence of yhp23 (yhp23) by immunoblotting for ER, using an ER-specific rabbit polyclonal antiserum.

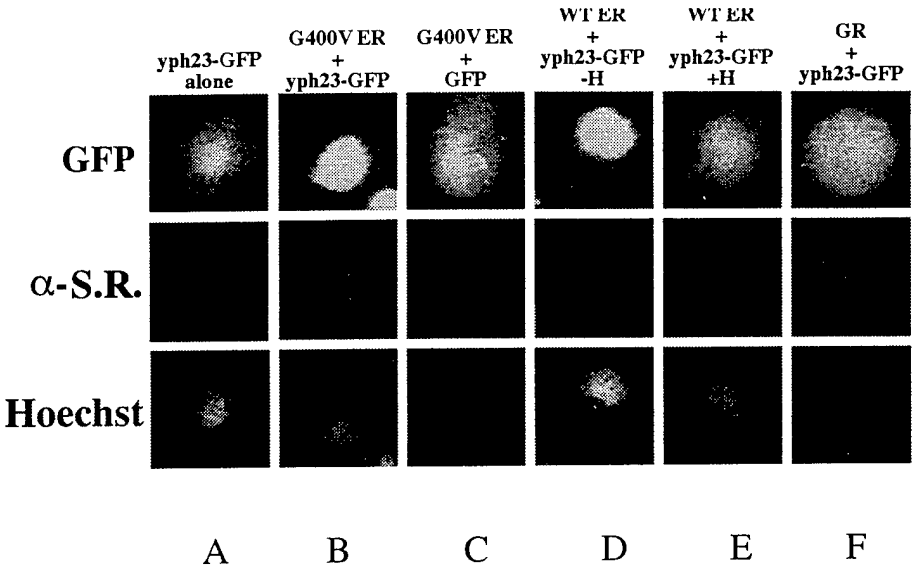

Figure 5. Co-localization of ER and yhp23.
To visualize yhp23 *in vivo*, a yhp23-green fluorescent protein (GFP) fusion protein was created by placing the GFP moiety onto the carboxy terminus of yhp23 (yhp23-GFP). The yeast strain W303a was transformed with a yhp23-GFP expression vector or GFP alone along with a galactose inducible vector expressing the wt ER and mutant G400V ER or GR. Cells were treated with 10^{-8}M β-estradiol (H+) or mock treated with ethanol vehicle (-H) and the distribution of GFP, ER or GR (α-SR) and the nucleus (Hoechst) were determined by fluorescent microscopy using a Zeiss Axioplan Fluorescent Microscope. A) The distribution of p23-GFP in the absence of any steroid receptor expression is largely cytoplasmic. B) Upon coexpression of G400V ER, p23-GFP becomes localized predominantly to the nucleus. C) Nuclear localization of GFP only was not seen when G400V ER was coexpressed, indicating that p23 is responsible for the localization of the fusion protein to the nucleus. D) Nuclear localization of yhp23-GFP was observed when yhp23-GFP was coexpressed with wt ER. E) No nuclear localization of yhp23-GFP was observed, when cells coexpressing wt ER and p23-GFP were incubated in 17-β-estradiol: the pattern of yhp23-GFP expression in these cells resembled that of yeast expressing no ER at all, suggesting that yhp23 is released upon estradiol binding. F) Coexpression of GR, a steroid receptor that exists outside the nucleus in the unbound state, did not result in nuclear localization of yhp23-GFP.

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Biochemistry

Regulation of estrogen receptor transcriptional enhancement by the cyclin A/Cdk2 complex

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ABSTRACT **We have found that ectopic expression of cyclin A increases hormone-dependent and hormone-independent transcriptional activation by the estrogen receptor *in vivo* in a number of cell lines, including HeLa cells, U-2 OS osteosarcoma cells and Hs 578Bst breast epithelial cells. This effect can be further enhanced in HeLa cells by the concurrent expression of the cyclin-dependent kinase activator, cyclin H, and cdk7, and abolished by expression of the cdk inhibitor, $p27^{KIP1}$, or by the expression of a dominant negative catalytically inactive cdk2 mutant. ER is phosphorylated between amino acids 82 and 121 *in vitro* by the cyclin A/cdk2 complex and incorporation of phosphate into ER is stimulated by ectopic expression of cyclin A *in vivo*. Together, these results strongly suggest a direct role for the cyclin A/cdk2 complex in phosphorylating ER and regulating its transcriptional activity.**

The estrogen receptor (ER) is a ligand-dependent transcriptional regulatory protein that controls the genetic programs affecting many aspects of cell growth and differentiation. In addition to ligand binding, phosphorylation plays an important role in regulating ER function. The receptor contains sites for both constitutive and ligand-dependent phosphorylation. Three serine residues (amino acids 104, 106, and 118) located within the N-terminal activation domain (AF-1) and one residue in the hinge region, S294, match the consensus sequence recognized by a family of serine/threonine proline-directed kinases that includes cyclin-dependent kinases (cdk), mitogen-activated protein kinases and glycogen synthase kinase-3. Ser-104, -106, and -118 are phosphorylated upon hormone treatment; serine to alanine mutations at these positions decrease ligand-dependent transcriptional activity (1–3). Accumulating evidence suggests that mitogen-activated protein kinase can phosphorylate Ser-118 and that this may lead to estradiol-independent ER activation or, alternatively, an increase in ligand-dependent ER activation. However, whether cdks target ER as a substrate for phosphorylation and affect its transcriptional activity remains unclear. Recently, a cdk-independent effect of cyclin D1 upon ER-dependent transcriptional activity was reported in T-47D breast cancer cells (4). A link between cdk enhancement of ER function and attendant receptor phosphorylation has not yet been demonstrated.

Cdks are a family of proteins composed of a regulatory cyclin subunit associated with a catalytic kinase subunit. The cyclin subunit appears to regulate subcellular localization and timing of activation as well as substrate specificity of the kinase complex. Cdk complexes regulate the activity of target molecules, including transcriptional regulatory proteins, by phosphorylation. Regulation of cdk activity is accomplished by proteins that activate (cdk activators or CAKs), or inhibit (cdk inhibitors or CDIs), kinase function (5–8). Because cdks control cell division, the dysregulation of cyclins, their kinase partners, and/or the upstream regulatory CAKs and CDIs, have been implicated in the initiation and promotion of hyperplasia and oncogenesis. In fact, the overexpression of the regulatory cyclin subunit and the dysregulation of the catalytic cdk subunit have been identified in a number of solid tumors, leukemias, and tumor-derived cell lines (9–18).

This study examines the effects of the cyclin A/cdk2 complex on ER transcriptional activation. We chose to focus on cyclin A for several reasons: (*i*) Cyclin A plays a multifaceted role in cell cycle progression and is a key regulator of cdk2, a serine/threonine proline-directed kinase with the potential to phosphorylate ER. (*ii*) Cyclin A expression is cell adhesion-dependent, such that its overexpression can lead to adhesion-independent cell growth, a hallmark of cellular transformation (19, 20). (*iii*) The synthesis and degradation of cyclin A are tightly regulated, suggesting that its aberrant expression could seriously jeopardize the control of cell growth (21, 22). (*iv*) Cyclin A overexpression has been implicated as an important indicator of oncogenesis in several contexts including human breast tumor-derived cell lines and a mouse mammary tumor virus breast cancer model (11, 13, 23–25). (*v*) Cyclin A shares several features with the protooncogene cyclin D1 including the ability to bind to and phosphorylate the retinoblastoma protein, such that inappropriate cyclin A expression leads to perturbations in the regulation of the G1 to S transition (26–28). (*vi*) Recent reports have also linked the degradation of $p27^{KIP1}$ (hereafter referred to as p27), an inhibitor of cyclin A/cdk2 activity, and aggressive breast and colorectal cancers (15, 17, 18). Together, these findings suggest that cyclin A may function as a protooncogene. To determine whether the cyclin A/cdk2 complex can affect ER function, we have examined the consequences of activation or inhibition of the cyclin A/cdk2 pathway on ER-dependent transcriptional activation.

MATERIALS AND METHODS

Plasmids. A FLAG epitope was added to the N terminus of the full-length wild-type ER cDNA. This construct was inserted into the pCMV-Neo^r (Invitrogen) expression vector; 0.5 μg DNA per 60-mm dish was used in the transfections. The vector pCDLSRα296 was used to express cyclin A, cyclin H, cdk7, cdk2, or the dominant negative mutant, cdk2TS. The pCMV5 plasmid expressed p27. Two micrograms of cyclin or cdk DNA was used for each 60-mm transfection plate, except in Fig. 2*C* where the amount of cyclin A-encoding plasmid was varied from 0.5 to 10.0 μg per dish. The ΔETCO reporter plasmid contained one estrogen response element upstream of the thymidine kinase promoter (-109) driving the expression of the chloramphenicol acetyltransferase (CAT) gene. This

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Abbreviations: ER, estrogen receptor; cdk, cyclin-dependent kinase; CAK, cyclin-dependent kinase activator; CDI, cyclin-dependent kinase inhibitor; CAT, chloramphenicol acetyltransferase; β-gal, β-galactosidase; GST, glutathione *S*-transferase.

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reporter lacks a nearby activator protein-1 binding site to ensure that the results obtained are not influenced by other regulatory elements in the plasmid. The vector pCMV-lacZ was used as an internal control to measure the efficiency of each transfection. The reporter- and β-galactosidase (β-gal)-encoding vectors were used at 2.0 and 0.5 μg DNA per 60-mm dish, respectively.

Mammalian Cell Culture and Treatments. The cell lines used in these studies were obtained from the American Type Culture Collection and maintained in DMEM (GIBCO/BRL) supplemented with 10% fetal bovine serum (HyClone), 50 units/ml each of penicillin and streptomycin (GIBCO/BRL), and 2 mM L-glutamine (GIBCO/BRL). Transfections were performed in phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum. For transfections, cells were seeded into 60-mm dishes at 2×10^5 cells per dish and transfected the following day by either the calcium phosphate precipitation or the liposome-mediated (Lipofectamine, GIBCO/BRL) methods (29). At 12–16 h posttransfection, cells were rinsed twice with PBS and refed with phenol red-free DMEM supplemented with 10% charcoal-stripped fetal bovine serum containing either 100 nM 17β-estradiol or the ethanol vehicle. CAT and β-gal assays were performed 24 h later as described (30). Protein expression was monitored by preparing whole cell extracts. Cells were lysed for 30 min on ice in 200 μl of high salt lysis buffer [400 mM NaCl/50 mM Tris·HCl, pH 8.0/0.5% Nonidet P-40/1 mM EDTA/1 mM DTT with protease inhibitors (1 μg/ml aprotinin/1 μg/ml leupeptin/1 μg/ml pepstatin A/1 mM phenylmethylsulfonyl fluoride)] and phosphatase inhibitors (1.0 mM NaF/10 mM β-glycerophosphate/1.0 mM sodium orthovanadate). Whole cell extract (100 μg) was separated by SDS/10% polyacrylamide gel and transferred to Immobilon paper (Millipore).

Glutathione *S*-Transferase (GST)-Protein Expression and Purification. Truncated versions of the human ER cDNA coding for amino acids 1–82, 1–115, and amino acids 1–121 were cloned into pGEX-5T-1 (Pharmacia). GST fusion proteins were expressed and purified as described (31).

Insect Cell Culture and Baculovirus Methods. High Five insect cells were maintained in Ex-Cell 405 Insect Culture Media (JRH Biosciences, Lenexa, KS) at 27°C. Baculovirus vectors (10^{-7} plaque-forming units) engineered to express human cyclin A or an hemagglutinin-tagged human cdk2 were used separately or in combination to infect cells. Cells (1×10^7cells per 100-mm dish) were infected with 0.5 ml of virus in a final volume of 3.0 ml for 2 h at 27°C and refed with 10 ml of Ex-Cell medium. Two days postinfection, cells were lysed on ice for 30 min in 0.5 ml of 120 mM NaCl, 50 mM Tris·HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 1 mM DTT with protease and phosphatase inhibitors as described above.

Immunoprecipitations. Insect cell immunoprecipitations were performed using ≈100 μg of extract and 5 μg of the mAb 12CA5 (Boehringer Mannheim) directed against the cdk hemagglutinin-epitope, or 5 μg of a human cyclin A-specific polyclonal antibody (#06–138, Upstate Biotechnology, Lake Placid, NY). Immune complexes were immobilized on protein A/G agarose beads (Santa Cruz Biotechnology), washed four times in 0.5 ml of lysis buffer and used in the *in vitro* kinase assay.

***In Vitro* Kinase Assays.** The GST-ER substrate (10 μg), ER 1–82, ER 1–115, or ER 1–121, was absorbed to 100 μl of a 50% slurry of glutathione-Sepharose 4B beads (Pharmacia) for 20 min at room temperature and washed twice with kinase buffer (50 mM potassium phosphate, pH 7.15/10 mM $MgCl_2$/5 mM NaF/4.5 mM DTT/1 mM phenylmethylsulfonyl fluoride). The immobilized substrate was added to the immunopurified kinase subunit(s) and incubated on ice for 5 min prior to the initiation of the kinase reaction in a final volume of 150 μl as described (31). The reaction products were separated by 12.5% SDS/PAGE, stained with Coomassie blue to visualize the receptor band, and autoradiography was performed from 5 to 30 min at room temperature. Aliquots of the reaction mixtures were also separated by SDS/PAGE and subjected to Western blot analysis to determine the levels of ER, cyclin A, and cdk2.

***In Vivo* Metabolic Labeling.** HeLa cells (1×10^6 cells per 100-mm dish) were transiently transfected with FLAG-ER and/or cyclin A and metabolically labeled with 1 mCi/ml (1 Ci = 37 GBq) of [^{32}P]orthophosphate in 2 ml of phosphate-free DMEM for 2 h at 37°C in the absence or presence of 100 nM 17β-estradiol. Cells were washed twice with PBS, placed on ice, and lysed directly on the plate by adding 200 μl of high salt lysis buffer. The *in vivo* labeled FLAG-tagged ER was immunopurified using 5 μg of the monoclonal anti-FLAG antibody (M2, Eastman Kodak). The ER protein recovered by immunoprecipitation was resolved on SDS/10% polyacrylamide electrophoresis gel, silver-stained, and dried. Autoradiography was performed for 12 h at room temperature to visualize the radiolabeled ER. The incorporated radioactivity was quantified using the National Institutes of Health IMAGE program to analyze the scanned autoradiogram and a digitized version of the silver stained gel.

RESULTS

Increased ER Transcriptional Enhancement by Ectopic Cyclin A Expression. To establish whether ectopic expression of cyclin A affects ER-dependent activation, we examined the ability of cyclin A to increase ER-mediated transcriptional enhancement. ER-deficient HeLa cells were transfected with an expression vector for the full-length human ER containing a FLAG epitope at its N terminus, the reporter plasmid estrogen response element-thymidine kinase-CAT, plasmids encoding human cyclin A and a constitutive β-gal expression vector as an internal transfection standard. Transfected cells were treated with 17β-estradiol or the ethanol vehicle for 24 h. Transcriptional activity was measured by CAT assay and normalized to β-gal activity. As shown in Fig. 1*A*, both hormone-dependent and hormone-independent ER transcriptional activity were increased roughly 3-fold when cyclin A is overexpressed. No effect of cyclin A on reporter gene activity was observed in the absence of ER (not shown). To ensure that this increased transcriptional activity was not a result of additional ER protein production, we monitored protein expression in whole cell extracts using Western blot analysis. As Fig. 1*B* illustrates, ER levels are not increased by cyclin A coexpression (compare lanes 5 and 6 to lanes 7 and 8). In addition, cyclin A is expressed above endogenous levels as a result of our transient transfection scheme and estradiol treatment does not alter cyclin A expression (Fig. 1*B*, compare lanes 1 and 2 to lanes 3 and 4). By increasing the amount of cyclin A used in these transfections, we were able to observe a concomitant increase in ER transcriptional activation (Fig. 1*C*). Coexpression of cyclin A and cdk2 also results in an increased ER-dependent transcriptional activity slightly above that of cyclin A alone. Expression of cdk2 alone, on the other hand, did not significantly alter the ER-dependent transcriptional activity (not shown). These findings suggest that cyclin A is a limiting factor for full hormone-dependent ER-mediated transcriptional enhancement, presumably by favoring the formation of active cyclin/cdk complexes from endogenous cdk2 subunits. Thus, cyclin A expression greatly magnifies the characteristic hormone-dependent ER transcriptional response, which suggests that this cyclin/cdk complex can act as an effector of the ER signaling pathway.

Reciprocal Effects of cdk Activators and Inhibitors on ER Transcriptional Enhancement. To further demonstrate that alterations in cyclin A/cdk2 activity can modify ER transcriptional enhancement, we used two classes of cdk regulatory proteins, CAK, which is composed of cyclin H and cdk7, and the CDI, p27. p27 inhibits many cyclin/cdk complexes, includ-

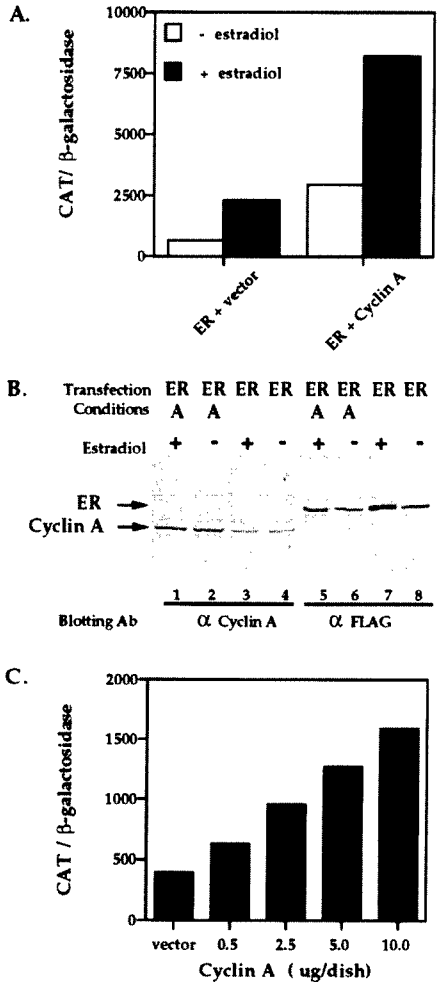

FIG. 1. Activation of ER transcriptional enhancement by ectopic cyclin A expression. (*A*) ER-deficient HeLa cells (2×10^5 cells per 60-mm dish) were transiently transfected using Lipofectamine with 2 μg of the estrogen response element containing reporter plasmid, possessing a single estrogen response element upstream of the thymidine kinase promoter fused to the CAT gene (ΔETCO), and 0.5 μg of the ER expression vector and 4 μg of the expression vector SRα296 (ER + vector) or 0.5 μg of the ER expression vector and 4 μg of SRα296-cyclin A (ER + cyclin A), along with 0.5 μg of pCMV-lacZ as an internal standard for transfection efficiency. Cells were incubated with 100 nM 17β-estradiol or the ethanol vehicle for 24 h as indicated, harvested and assayed for CAT and β-gal activity. (*B*) ER and cyclin A expression in transfected HeLa cells. Whole cell extracts were prepared from a parallel set of transfected cells. Equal amounts of protein (100 μg per lane) were separated by SDS/10% polyacrylamide gel, transferred to Immobilon paper, probed with the M2 monoclonal antibody directed against the FLAG-epitope on ER or a polyclonal antibody against human cyclin A, and visualized with an alkaline phosphatase-conjugated goat secondary antibody. (*C*) Increasing amounts of cyclin A lead to increased ER transcriptional activity. Using the calcium phosphate procedure, HeLa cells were transiently transfected with increasing amounts of cyclin A (0.5 μg to 10.0 μg) with a constant amount of ER expression and reporter plasmids, and CAT activity was measured in the presence of 17β-estradiol. For transfection experiments, data represent the mean of at least two experiments done in duplicate with <10% variation.

ing cyclin A/cdk2, cyclin E/cdk2, and cyclin D/cdk4 (32, 33). We were particularly interested in studying p27 in light of recent reports (15, 17, 18) linking its premature or excessive degradation to aggressive breast and colorectal cancers.

Fig. 2*A* illustrates that cdk activation by expression of cyclin A/cdk2 or CAK (cyclin H and cdk7) leads to a greater than 2-fold increase in both hormone-dependent and hormone-independent ER transcriptional activity. The coexpression of

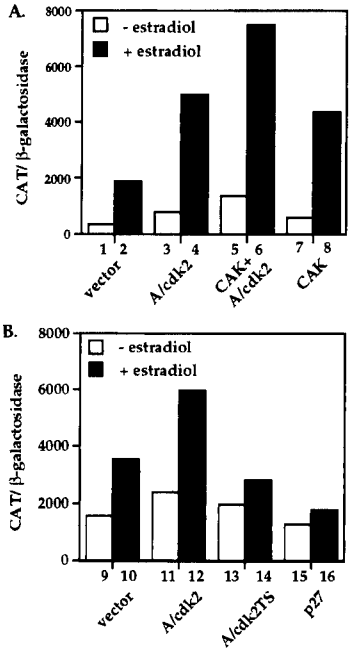

FIG. 2. Effects of cdk activators and inhibitors on ER transcriptional enhancement. (*A*) Effects of CAK (cyclin H/cdk7) on ER transcriptional activation. HeLa cells were transiently transfected using the calcium phosphate procedure with paired ER expression and reporter plasmids as described in Fig. 1, along with a control empty expression vector, (lanes 1 and 2); or expression vectors for cyclin A/cdk2 (lanes 3 and 4); cyclin A/cdk2 + CAK (lanes 5 and 6) and CAK alone (lanes 7 and 8). (*B*) Effects of cdk inhibitors on ER transcriptional activation. HeLa cells were transiently transfected with ER expression and reporter constructs along with an empty expression vector (lanes 9 and 10); or expression vectors for cyclin A/cdk2 (lanes 11 and 12); cyclin A/cdk2TS (dominant negative) (lanes 13 and 14) and p27 (lanes 15 and 16). Hormone treatment and activity assays were performed as described in Fig. 1. Data represent the mean of two experiments done in duplicate with <10% error.

all four proteins, the cyclin/cdk complex as well as the CAK complex, further augments (4-fold) this response and lends further support for cyclin/cdk involvement in the regulation of ER-dependent transcriptional activity.

We next asked if a decrease in cdk activity would reduce ER-dependent transcriptional activation. We chose two means of inhibiting cdk2 activity. Initially, the CDI, p27, was ectopically expressed in HeLa cells and ER-dependent transcriptional enhancement was measured. Ligand-dependent and, to a lesser degree, ligand-independent transcriptional activation by ER was reduced by p27 expression (Fig. 2*B*). This effect is noted in either the presence or absence of ectopically expressed cyclin A. Therefore, reducing cdk activity leads to impaired ER transcriptional activity.

At this point, we could not discriminate between an effect of p27 upon cdc2, cdk2, or cdk4, since p27 can inhibit all of these kinases. Therefore, we sought another means of reducing cdk2 activity by using a catalytically inactive cdk2 mutant to specifically block endogenous cdk2 activity. This cdk derivative, designated cdk2TS, is competent for cyclin A binding, but it cannot bind to ATP due to two consecutive amino acid changes in the ATP-binding site (Lys-33 and -34 are replaced by threonine and serine, respectively). This mutant acts as a dominant negative by sequestering cyclin A, thereby preventing it from binding and activating endogenous wild-type cdk2.

By expressing the dominant negative cdk2 mutant, we were able to reduce significantly the ER response to ligand treatment (Fig. 2*B*). Ectopic expression of a dominant negative cdc2 mutant had little effect on ER activity (not shown). These results strongly argue that the observed decrease of ER

transcriptional activity by p27 is due to inactivation of cdk2 and further suggests the importance of cyclin A/cdk2 enzymatic activity for hormone-dependent transcriptional enhancement by ER. It appears then, that the balance among the cdk regulatory proteins, cyclins, CAK, and CDIs, is critical in determining ER transcriptional activity.

Phosphorylation of ER by the Cyclin A/cdk2 Complex. Next, we investigated whether the cyclin A/cdk2 complex can phosphorylate ER. To determine if ectopic expression of cyclin A increased the amount of phosphate incorporated into ER *in vivo*, HeLa cells were transfected with ER alone or in combination with cyclin A and cells were metabolically labeled with [^{32}P]orthophosphate for 2 h in the presence or absence of 17β-estradiol. For each sample, the total amount of ER visualized by silver staining was used to standardize the amount of incorporated ^{32}P. The untreated ER condition was arbitrarily set as 1. As shown in Fig. 3*A*, ER phosphorylation is increased by ectopic expression of cyclin A in both the absence (3×) and presence (3.7×) of hormone. Thus, the presence of cyclin A increases incorporation of phosphate into ER by activating endogenous cdks.

To further investigate the effect of cyclin A/cdk2-dependent phosphorylation of ER we performed *in vitro* kinase assays. Three ER derivatives, containing amino acids 1–82, 1–115 or 1–121 were bacterially expressed and purified as GST-fusion proteins and used as substrates for phosphorylation by immunopurified baculovirus-expressed cyclin A and cdk2. These particular derivatives were chosen since the ER 1–121 derivative contains three serine-proline motifs at Ser-104, -106, and -118, whereas ER 1–115 contains only Ser-104 and -106. ER 1–82 lacks all of the putative serine-proline phosphorylation sites and thus serves as a negative control. Fig. 3*B* demonstrates that both ER 1–121 and ER 1–115 were phosphorylated by the cyclin A/cdk2 complex but not by either subunit alone. On the other hand, ER 1–82 was not phosphorylated by the cyclin A/cdk2 complex. In each reaction, expression of the ER substrate and the kinase subunits was verified by Western blotting and found to be identical (not shown). The fact that ER 1–121 and ER 1–115 derivatives were phosphorylated while ER 1–82 was not, strongly suggests that the residues contained in the region comprised by amino acids 83–121 comprise a motif targeted by the cyclin A/cdk2 complex. This data provide *in vitro* biochemical evidence that ER is a substrate for cyclin A/cdk2-dependent phosphorylation.

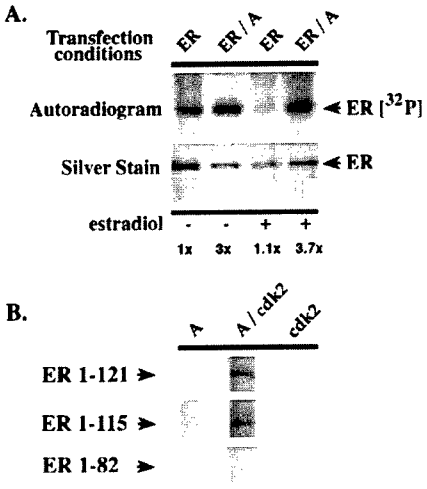

FIG. 3. ER phosphorylation by cyclin A/cdk2. (*A*) Phosphorylation of ER *in vivo* in the absence and presence of cyclin A. HeLa cells were transfected with FLAG-ER in the absence and presence of cyclin A expression vector using the Lipofectamine method as described in Fig. 1 and metabolically labeled with [^{32}P]orthophosphate as described in *Materials and Methods*. Whole cell extracts were prepared and ER was immunoprecipitated using the FLAG-mAb, M2. ER immunoprecipitates were separated by SDS/10% polyacrylamide gel, silver-stained (*Lower*), and exposed to film to visualize the phosphorylated receptor (*Upper*). The incorporated radioactivity was normalized to the amount of ER immunoprecipitated in each condition. The value of the untreated ER was arbitrarily set as 1. (*B*) Phosphorylation of ER *in vitro* by the cyclin A/cdk2 complex. Bacterially expressed GST ER 1–82, 1–115, and 1–121 derivatives were absorbed onto glutathione agarose beads and used as substrates for *in vitro* kinase assays. Cyclin A and cdk2 were produced in 5B insect cells, separately or in combination, purified by immunoprecipitation, and used in the kinase assays as described in *Materials and Methods*. The proteins were separated by SDS/10% polyacrylamide gel and the phosphorylated products were visualized by autoradiography.

Increased ER Transcriptional Enhancement in Response to Ectopic Cyclin A Expression in Multiple Cell Lines. To test our hypothesis that the regulation of ER-dependent transcriptional activity by the cyclin A/cdk2 complex is not specific to HeLa cells but rather reflects a general mode of regulation, we repeated our transcriptional activity assay in a variety of cell lines. We tested Hs 578Bst cells derived from breast tissue peripheral to an infiltrating ductal carcinoma and an ER-negative osteosarcoma cell line, U-2 OS. Cells were transiently transfected as described above, treated with 17β-estradiol or the ethanol vehicle for 24 h and transcriptional activity was measured. In the three cell types utilized, we observed a significant increase in ER ligand-dependent and -independent transcriptional activation (Fig. 4). These data imply that the ability of a cyclin A/cdk2 complex to enhance ER ligand-dependent transcription is conserved across multiple cell types.

DISCUSSION

We have examined the effects of cyclin A/cdk2 activation and inhibition on ER-dependent transcriptional enhancement. Here, we provide evidence that alterations in the regulation of the cyclin A/cdk2 complex lead to changes in both hormone-independent and hormone-dependent ER transcriptional enhancement. Our findings indicate that the ectopic expression of cyclin A elevates ER transcriptional activity and that this effect is not restricted to a single cell type. Our results likely represent an underestimate of the full impact of cyclin A on ER transcriptional activity, since these findings are obtained in cells that contain endogenous cyclin A and cdk2. Consistent with this view, ER transcriptional activity in both the presence and absence of hormone is virtually abolished under conditions where cyclin A/cdk2 activity is suppressed by the kinase inhibitor p27, or by a dominant negative cdk2 mutant. Thus it appears that cyclin A, the regulatory subunit of cdk2, is a limiting cofactor in the regulation of ER-dependent transcriptional activation in the cells examined.

Our findings also demonstrate that ER is a substrate for cyclin A/cdk2. ER is phosphorylated *in vitro* by cyclin A/cdk2 complexes and incorporation of phosphate into ER is stimulated by cyclin A expression *in vivo*. Importantly, our biochemical data demonstrate that the presence of three putative cdk target sites (Ser-104, Ser-106, Ser-118) is correlated with cyclin A/cdk2-dependent phosphorylation of the ER substrate *in vitro*. Work is ongoing to identify the precise residue(s) on the receptor phosphorylated by the cyclin A/cdk2 complex. Together, these data suggest that the cyclin A/cdk2 complex directly phosphorylates ER and that this modification serves to increase the receptor's transcriptional regulatory properties.

Zwijsen *et al.* (4) observed that overexpression of cyclin D1 increased ER-dependent transcriptional activation in T-47D cells without direct ER phosphorylation by cyclin D1/cdk4. Cyclin D1 appears to act indirectly as an ER cofactor, perhaps by tethering or phosphorylating other regulatory proteins that effect downstream signaling by ER. In contrast, we observed the cyclin A/cdk2 complex directly acting upon the receptor leading to its phosphorylation and a marked increase in

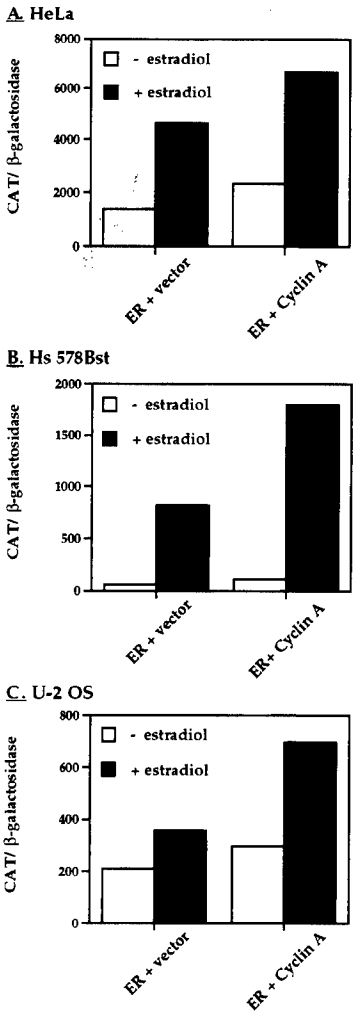

FIG. 4. Cyclin A enhances ER-dependent transcriptional activation in multiple human cell lines. Three ER-negative cell lines, (*A*) HeLa cells derived from a human cervical carcinoma, (*B*) Hs 578Bst, a human cell line derived from normal breast tissue, and (*C*) U-2 OS, a human osteosarcoma cell line, were transfected with ER expression and reporter plasmids as described in Fig. 1. Cells were also cotransfected with the empty expression vector or the expression vector encoding cyclin A. Hormone treatment and activity assays were performed as described in Fig. 1. Results shown represent a single experiment done in duplicate whose error was <10%. This experiment was repeated twice more with similar results.

ER-dependent transcriptional activity. Zwijsen *et al.* (4) did not observe an effect of cyclin A overexpression on ER-dependent transcriptional activation in T-47D cells and conversely, we failed to detect an increase in ER activity when cyclin D1 was ectopically expressed in either HeLa or ER-expressing U-2 OS human osteosarcoma cell line (J.M.T. and M.J.G., unpublished data). One factor contributing to the observed differences may lie in the cell types used in these studies. Recent reports and our own observations suggest that the level of expression of CDIs, such as p27, differ dramatically among cell types (34). Given that these proteins function as kinase inhibitors and as recently recognized cyclin D/cdk4 assembly factors, differences in CDI expression might significantly alter cdk signaling (34, 35). Among several breast cancer cell lines tested, T-47D cells were found to express high levels of p27 (34). This finding may account for the lack of a cyclin A effect in these cells, since the resulting cyclin A/cdk2 complex will be inhibited by endogenous p27. In contrast, HeLa cells used in this study express comparatively low amounts of p27, making ER-dependent transcription more sensitive to ectopic cyclin A expression. The cell-specific differences in the level of endogenous p27 may also help explain the ability of cyclin D1 to activate ER in T-47D cells, but not in HeLa cells, since abundant p27 may favor the formation of a cyclin D1/cdk4 complex, which may in turn phosphorylate an ER coactivator, or facilitate complex formation between ER and a receptor cofactor. Examination of the consequences of ectopic cyclin A expression in several breast cell lines has revealed an inverse correlation between cyclin A activation of ER transcriptional enhancement and the level of endogenous p27 (J.M.T. and M.J.G., unpublished data). Thus, the level of endogenous CDI may determine which cyclin isotypes will affect ER transcriptional activity, and may account for the observed differences between our findings and that of the Zwijsen *et al* (4).

Based on our findings, we propose a model for ER regulation by the cyclin A/cdk2 complex (Fig. 5). The cyclin A/cdk2 complex directly phosphorylates the receptor and in doing so, facilitates its interaction with the basal transcriptional machinery or an ER coactivator, which increases the receptor's ability to activate transcription. Inhibition of cdk activity by CDIs, such as p27, or through a reduction in cyclin or cdk expression, would decrease receptor phosphorylation, weakening these putative ER-transcription factor contacts, thus leading to decreased receptor transcriptional activity. We further envision that the expression of the CAK complex, cyclin H and cdk7, enhances ER transcriptional activation by increasing the activity of the endogenous cyclin A/cdk2 pool. Since cyclin H and cdk7 are also components of TFIIH (5, 36–38), we cannot exclude the possibility that this complex may be acting at the level of TFIIH to increase its catalytic activity, which in turn, increases ER transcriptional activity. Together, these data suggest that the cyclin A/cdk2 complex directly influences ER's transcriptional regulatory properties. We conclude that ultimately the balance of these cdk regulatory proteins determines kinase activity, which in this case translates into differential transcriptional activation by ER.

A complex picture of signal transduction by ER is emerging that appears to rely on the collaboration of multiple factors for its regulation, with each event in the pathway vulnerable to subversion. This subversion may take the form of aberrant expression of cyclin or cdk subunits, or CDIs, leading to alterations in receptor phosphorylation and activity that might contribute to uncontrolled cell proliferation. Clearly, the involvement of cyclins, cdks, CAKs, and CDIs in ER-mediated

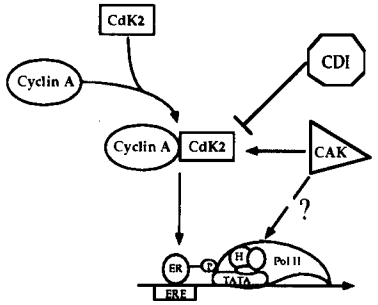

FIG. 5. Model for regulation of ER-dependent transcriptional activation by the cyclin A/cdk2 complex. The cyclin A/cdk2 complex phosphorylates ER which increases the receptor's ability to activate transcription by facilitating its interaction with the basal transcriptional machinery or an ER coactivator. Inhibiting cdk activity by CDIs has the opposite effect, resulting in reduced ER phosphorylation and decreased receptor transcriptional activity. Expression of the CAK complex, cyclin H, and cdk7, enhances ER-dependent transcription by increasing the activity of the endogenous cyclin A/cdk2 pool. It is also conceivable that the CAK complex may be acting at the level of TFIIH to increase ER transcriptional enhancement. We conclude that it is the balance among the cyclins, cdks, and their regulatory proteins that will ultimately determine ER transcriptional activity.

transcriptional regulation is complex and will require further investigation. It is likely, that phosphorylation events mediated by the cyclin/cdk pathway will emerge as a general mechanism of controlling steroid hormone action (31, 39).

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